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Genotyping Hepatitis C Viruses from Southeast Asia by a Novel Line Probe Assay That Simultaneously Detects Core and 5′ Untranslated Regions[▽]

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Hepatitis C viruses (HCVs) display a high level of sequence diversity and are currently divided into six genotypes. A line probe assay (LiPA), which targets the 5′ untranslated region (5′UTR) of the HCV genome, is widely used for genotyping. However, this assay cannot distinguish many genotype 6 subtypes from genotype 1 due to high sequence similarity in the 5′UTR. We investigated the accuracy of a new generation LiPA (VERSANT HCV genotype 2.0 assay), in which genotyping is based on 5′UTR and core sequences, by testing 75 selected HCV RNA-positive sera from Southeast Asia (Vietnam and Thailand). For comparison, sera were tested on the 5′UTR based VERSANT HCV genotype assay and processed for sequence analysis of the 5′UTR-to-core and NS5b regions as well. Phylogenetic analysis of both regions revealed the presence of genotype 1, 2, 3, and 6 viruses. Using the new LiPA assay, genotypes 6c to 6l and 1a/b samples were more accurately genotyped than with the previous test only targeting the 5′UTR (96% versus 71%, respectively). These results indicate that the VERSANT HCV genotype 2.0 assay is able to discriminate genotypes 6c to 6l from genotype 1 and allows a more accurate identification of genotype 1a from 1b by using the genotype-specific core information.

Hepatitis C virus (HCV), an enveloped positive-stranded RNA virus of the family *Flaviviridae*, is recognized as a major cause of chronic liver disease. Because of its high genetic heterogeneity, HCV has been classified into six genotypes and a huge number of subtypes (18, 20). Genotypes 1, 2, and 3 are widely distributed around the world, whereas genotypes 4 and 5 have been identified mainly in Africa (22). Genotype 6 was found locally in Southeast Asia (SEA) (11, 26, 27, 28).

Genotype identification is clinically important for prediction of responses to, and in determining the duration of, antiviral therapy (30). This is illustrated by the fact that genotypes 1 and 4 are more resistant to treatment with pegylated alpha interferon and ribavirin than genotypes 2 and 3 (9, 31). Moreover, it has been suggested that patients with chronic HCV genotype 1b infection show more severe liver disease than patients infected with other genotypes (17). Nowadays, most treatment protocols require preceding genotype information for HCV-infected patients.

A variety of technologies has been developed for HCV genotype determination. Most of these assays rely on the amplification of short HCV RNA regions from clinical specimens, followed by a type-specific assay, such as line probe reverse hybridization (23, 25), restriction fragment length polymorphism analysis (1), or sequence analysis (20, 29). Almost all available commercial assays target the 5′ untranslated region (5′UTR), because the highly conserved sequences of this region are most suitable for reverse transcription-PCR (RT-PCR) amplification.

The VERSANT HCV genotype assay (LiPA) is one of the most widely used methods for HCV genotyping. In this assay the 5′UTR of HCV is amplified with biotinylated primers, after which the PCR product is hybridized to a membrane impregnated with genotype-specific probes and detected with streptavidin linked to a colorimetric detector (24). Despite the high conservation of the 5′UTR, genotype determination of HCV based on the 5′UTR is accurate for most genotypes (8, 21, 29). However, it has been noted that methods that are based on the use of the 5′UTR falsely identify genotypes 6c to 6l from SEA as genotype 1, which is also the case in the VERSANT HCV genotype assay (3, 24). Moreover, this assay is unable to distinguish genotype 1a from 1b in 5 to 10% of the cases (2, 21). Therefore, the use of other coding regions of the HCV genome (e.g., core, E1, and/or NS5b) has been recommended for genotype identification (18).

A new generation of the line probe assay (VERSANT HCV genotype 2.0 assay) was recently developed that uses core sequence information, in addition to 5′UTR, to improve the accuracy of HCV genotyping. After amplification of the 5′UTR-to-core region and hybridization to type-specific probes of the 5′UTR and core, it is possible to distinguish between genotypes 1a, 1b, and 6c to 6l. In the present study, we selected 75 sera of HCV RNA-positive blood donors collected

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from Bangkok, Thailand, and Ho Chi Minh City, Vietnam, in order to test the accuracy of the new VERSANT HCV genotype 2.0 assay. The data were compared to results obtained with the previous VERSANT HCV genotype method and sequence analysis. We show that the VERSANT HCV genotype 2.0 assay is able to discriminate genotypes 6c to 6l from genotype 1 and allows a more accurate identification of genotype 1a from 1b by using the genotype-specific core information.

MATERIALS AND METHODS

Samples. A total of 152 HCV-positive plasma samples of blood donors from Ho Chi Minh City, Vietnam, and Bangkok, Thailand, were obtained during the period from 2000 to 2002. From this collection of samples, 75 were selected based on an initial characterization to include preferentially genotype 6 viruses (12, 26). Samples were kept at −80°C until further analysis.

RNA isolation and RT-PCR. Viral RNA was isolated from 200 μl of serum or EDTA-plasma by the High-Pure RNA isolation kit (Roche, Diagnostics GmbH) and eluted with 50 μl of water. cDNA was synthesized by using 10 μl of extracted RNA, 2 μl of random hexamer primers (150 μg/ml; Boehringer Mannheim) and Moloney murine leukemia virus reverse transcriptase (Invitrogen) according to the instructions of the manufacturers. The 5′UTR-core sequences (nucleotides 47 to 695, according to the numbering system for reference strain HCV-H GenBank accession number M67463) were amplified by nested PCR with the primers 16 (5′-GRGGCGACACTCCACCAT-3′) and 410 and the primers s17 and 951, as described previously (10). NS5b sequences (nucleotides 8283 to 8624, according to the numbering system for HCV-H strain) were amplified by nested PCR with primers Pr3, Pr4, Pr1, and Pr2 using conditions described in previous reports (16) with modifications. Briefly, in the first PCR, performed with Pr3 and Pr4, 1.5 mM $MgCl_2$ was used in the reaction mixture, and the following thermal profile: initial denaturation at 94°C for 90 s; five cycles of denaturation at 94°C for 30 s, annealing at 64°C for 45 s, and elongation at 72°C for 1 min; followed immediately by 30 cycles at 94°C for 30 s, 64°C with a drop of −0.5°C between each cycle for 45 s and elongation at 72°C for 1 min. The last five cycles were performed at 94°C for 30 s, 48°C for 45 s, and 72°C for 1 min. A final elongation at 72°C for 10 min was also included. The nested PCR, performed with primer Pr1 and Pr2, was carried out on 2 μl of the first PCR product with the following thermal profile: 95°C for 7 min; 50 cycles at 95°C for 30 s, 63°C for 30 s, and 72°C for 30 s, with a final elongation step at 72°C for 10 min. The amplified products were gel purified and sequenced.

DNA sequencing and phylogenetic analysis. Sequence reactions on PCR products were performed by using BigDye terminator cycle sequencing ready reaction kit (Applied Biosystems) and an ABI Prism 3100 autosequencer (Applied Biosystems). Multiple sequence alignments were generated with BioEdit version 7.0.1. Phylogenetic analyses were conducted by using the Tajima-Nei model with rate heterogeneity parameter and the neighbor-joining method. Confidence values were calculated by bootstrap analysis (1,000 replicates), and consensus trees were produced by using MEGA version 3.0 (7).

The reference strains used in the present study were obtained from GenBank: genotype 1a (M67463), 1b (AB016785), 1c (AY051292), 2a (AB047639), 2b (AB030907), 2c (D50409), 2k (AB031663), 3a (AF046866), 3b (D49374), 3k (10a, D63821), 4a (D45193, Y11604), 5a (D50466, Y13184), 6a (D88469, AY859526), 6b (D84262), 6c (7d, D37843, D37885), 6d (7b, D84263), 6e (7a, D31971, D30397), 6f (7c, D38078, D38078), 6g (11a, D63822), 6 h (9a, D84265), 6i (9b, D37850, D37864), 6j (9c, D37848, D37862), 6k (8b, D84264), 6l (8a, D88470, D87357), and 6n (DQ278894).

INNO-LiPA line probe assay. Sera samples were extracted with the viral DSP kit (QIAGEN) according to the manufacturer's instructions, with an elution volume of 60 μl. The cDNA synthesis and amplifications for VERSANT HCV genotype assay (Bayer HealthCare, manufactured by Innogenetics, Ghent, Belgium) were performed according to the manufacturer's instructions. For the VERSANT HCV genotype 2.0 assay (Bayer HealthCare, manufactured by Innogenetics) a multiplex RT-PCR was performed according to the manufacturer's instructions on the extracts to amplify the 5′UTR and core regions of the HCV genome, utilizing the primers HCVPr95 AT&AC, HCVPr96b, HCVPr769b, and HCVPr822b, generating two distinct PCR products from 5′UTR and core regions, respectively. Briefly, master mixes were prepared by using these primers, buffer, enzymes, and deoxynucleoside triphosphates, including dUTP, from the VERSANT HCV amplification 2.0 assay. Twenty microliters of RNA extract was added to the master mix and treated with uracil-*N*-glycosylase to prevent contamination by the amplification product from previous PCRs. Subsequently, the mix was incubated for 30 min at 50°C and 15 min at 95°C for RT and PCR activation, respectively, immediately followed by 40 cycles of 95°C for 30 s, 50°C for 30 s, and 72°C for 15 s. After amplification, the PCR products were immediately stored at −20°C to prevent residual uracil-*N*-glycosylase activity. Hybridization and color development steps were performed in the Auto-LiPA48 instru-

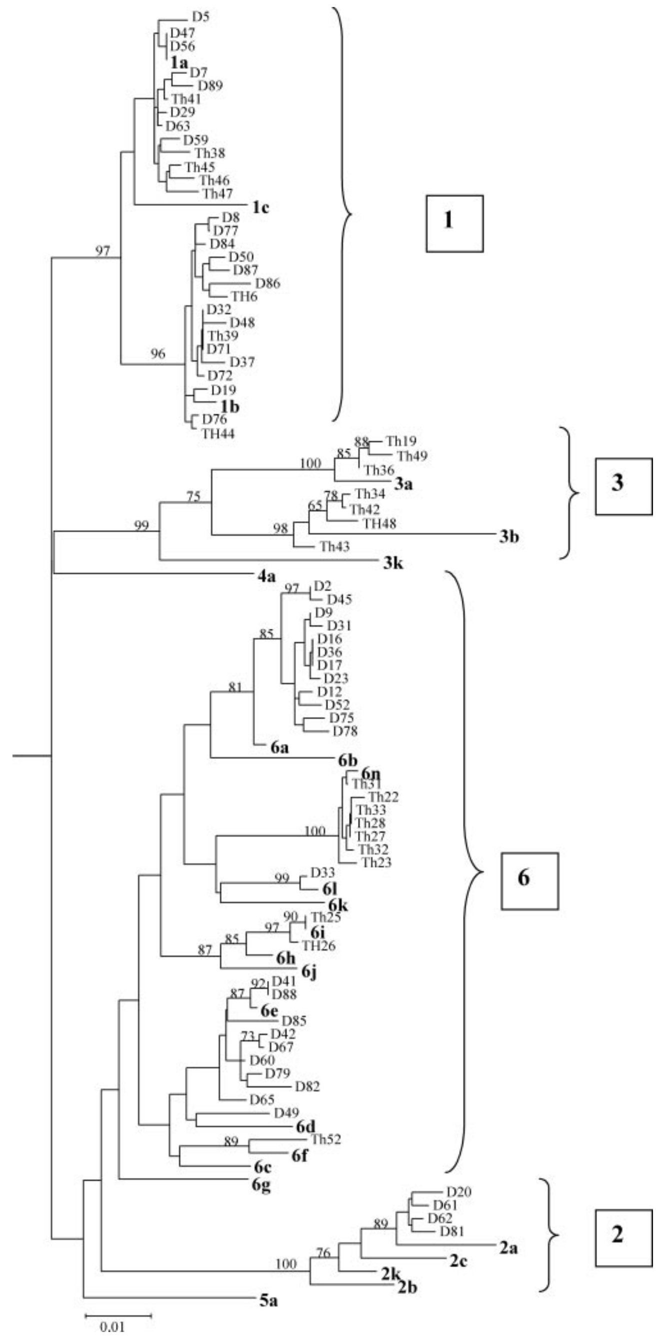

FIG. 1. Midpoint rooted neighbor-joining tree of the 5′UTR-to-core region among selected HCV field viruses from SEA and prototype strains. The tree was constructed for nucleotides 47 to 695 of the HCV genome (numbering is according to reference strain HCV-H GenBank accession number M67463) by using the Tajima-Nei model with rate heterogeneity parameter. Confidence values (>70%) calculated by bootstrap analysis (1,000 replicates) are indicated at the major branching points. Branch lengths are drawn to scale. The prototype HCV strains obtained from GenBank are indicated in boldface.

TABLE 1. Comparison of HCV genotyping results obtained by phylogenetic analysis, the VERSANT HCV genotype assay (5′UTR), and the VERSANT HCV genotype 2.0 assay (5′UTR-core) for 73 specimens obtained from SEA

Genotype	No. of HCV genotyped samples (type or subtype) as determined by:[a]			
	Phylogenetic analysis		VERSANT HCV (5′UTR)	VERSANT HCV 2.0 (5′UTR-core)
	5′UTR-core	NS5B		
Genotype 1				
Subtype 1a	13	13	6 (1a) 2 (1a or 1b) 1 (1b) 4 (1)	11 (1a) 2 (1)*
Subtype 1b	16	16	14 (1b) 2 (1)	14 (1b) 2 (NT)*
Genotype 2				
Subtype 2a	4	4	3 (2a or 2c) 1 (2)	4 (2a or 2c)
Genotype 3				
Subtype 3a	3	3	3 (3a)	3 (3a)
Subtype 3b	4	4	3 (3b) 1 (3)	3 (3b) 1 (3)*
Genotype 6				
Subtype 6a	12	12	12 (6a)	12 (6a or 6b)
Subtypes 6c to 6n	21	21	19 (1b) 2 (1)	20 (6c to 6l) 1 (NT)*

[a] NT, nontypeable. *, INNO-LiPA line patterns shown in Fig. 2C.

ment (Innogenetics) according to the manufacturer's instructions and were not different for the two assays. Strip patterns obtained from both assays were interpreted according to the manufacturer's interpretation chart. HCV genotyping results from the LiPA assays were compared to the gold standard phylogenetic analysis of core and NS5b sequences. The sensitivity of the assay was determined by probit analysis to 2,106 IU/ml.

Nucleotide sequence accession numbers. New sequences reported in the present study have been submitted to GenBank database and assigned accession numbers DQ155445, DQ155447 to DQ155455, DQ155457, DQ15460 to DQ15463, DQ15465 to DQ15474, DQ155477 to DQ15493, DQ155495 to DQ15500, and DQ640336 to DQ640361 for 5′UTR-to-core phylogenetic analysis. Sequences accession numbers DQ155503, DQ155505 to DQ155513, DQ155515, DQ155518 to DQ155521, DQ155523 to DQ155532, DQ155535 to DQ155551, DQ155553 to DQ155558, and DQ640362 to DQ640386 were used for NS5b sequences analysis.

RESULTS

Determination of HCV genotype by sequence analysis. To investigate the accuracy of the newly developed VERSANT HCV genotype 2.0 assay for genotype 6 identification, we selected a set of samples from SEA HCV-positive blood donors to encompass mainly genotype 1 and 6 viruses. A total of 75 samples, 50 from Vietnam and 25 from Thailand, were included in the present study. Viral RNA served as a template for RT-PCR amplification of 5′UTR-core (nucleotides 47 to 695) and NS5b (nucleotides 8283 to 8624) sequences. Amplicons were sequenced twice in both orientations. Degenerate sequences were preserved in order to determine the specificity of the line probe hybridization assays (see below). To determine the HCV genotype, phylograms were constructed with reference strains for the 5′UTR-core and NS5b regions (Fig. 1 and data not shown). Only samples that showed genotype consistency of both regions were selected (n = 73), and two discordant samples (D3 and D54), which turned out to be a recombinant virus and a mixed infection, were characterized by full-length genome analysis as described elsewhere (12). As evident from the 5′UTR-core phylogenetic analysis (Fig. 1 and Table 1), our sample collection contained genotype 1 (n = 29), 2 (n = 4), 3 (n = 7), and 6 (n = 33) viruses.

Determination of HCV genotype by the VERSANT HCV genotype (5′UTR) assay. To examine the accuracy of HCV genotyping by the line probe hybridization assay based on the 5′UTR sequence, we tested all selected samples using the VERSANT HCV genotype assay (Fig. 2A and Table 1). This assay correctly determined the genotype of 71% (52 of 73) of

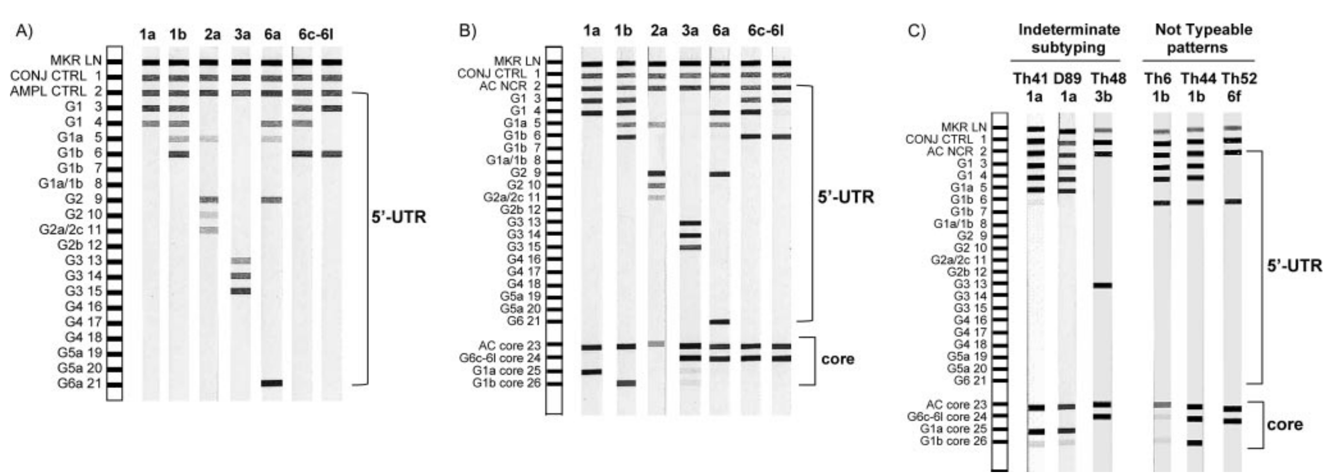

FIG. 2. (A and B) Comparison of INNO-LiPA strip patterns obtained from VERSANT HCV genotype assay (5′UTR lines) (A) and the new VERSANT HCV genotype 2.0 assay (5′UTR/core lines) (B). Both assays were tested with HCV genotype 1a, 1b, 2a, 3a, 6a, and 6c to 6l samples. Type-specific line numbers and interpreted genotypes of 5′UTR and core regions are indicated on the left side of each panel. (C) Patterns of the VERSANT HCV genotype 2.0 assay obtained from subtype-indeterminate samples (from Th41, D89 [genotype 1a], Th48 [genotype 3b]) and from nontypeable samples (from Th6, Th44 [genotype 1b], and Th52 [genotype 6f]). Sample codes and specific probe numbers are indicated. MKR, marker line; CONJ CTRL, conjugate control; AMPL CTRL, amplification control; AC NCR, amplification control of noncoding region; AC CORE, amplification control of core.

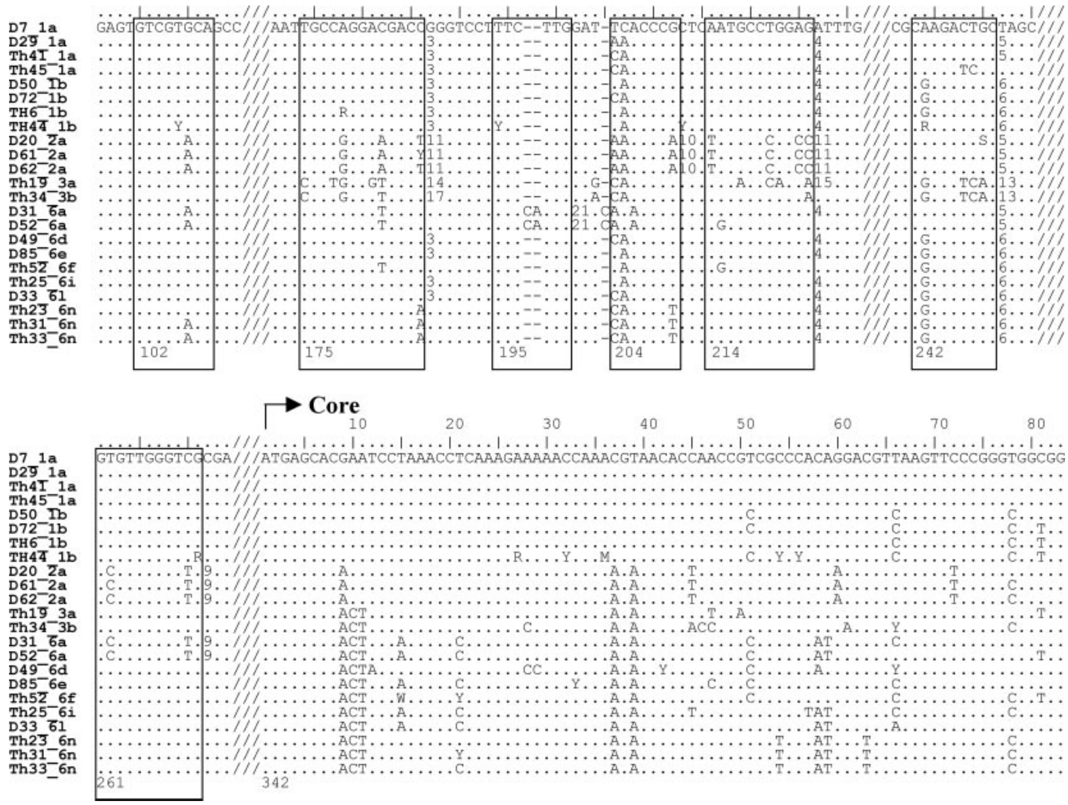

FIG. 3. Alignment of the 5′UTR and core sequences of a subset of samples obtained from SEA. Sample codes and genotypes are indicated in the left-hand column. Residues identical to the major sequence are indicated by dots. The positions of specific probes of the VERSANT HCV genotype assay (5′UTR) are boxed. The first nucleotide position of each box is indicated in the bottom left corner. The LiPA line number outside the box indicates the predicted reactivity of each isolate in the sequence alignment. The core sequence alignment starts with the ATG initiation codon (positions 342 to 345 of the HCV genome; the numbering system is as described for Fig. 1). Slashes indicate discontinuous sequences within the 5′UTR.

the samples compared to sequence analysis. From these identified genotypes, 15% (8 of 52) were subtype indeterminate: 4 were subtype 1a, 2 were subtype 1b, 1 was subtype 2a, and 1 was subtype 3b. Only one sample (2%) of genotype 1a was incorrectly subtyped as genotype 1b (Table 1). All 21 samples that could not be genotyped correctly are genotypes 6c to 6l that were typed to genotype 1. The 5′UTR sequences of the 6c to 6l samples are very similar to the genotype 1 5′UTR sequences, especially in the regions where probes 3, 4, and 6 are located (24) (Fig. 3), which explains the mistyping of the genotypes 6c to 6l in this assay.

Determination of HCV genotype by the VERSANT HCV genotype 2.0 (5′UTR-core) assay. The new HCV LiPA assay consists of 25 lines in total (Fig. 2B). Lines 1 to 21 are identical to the strip configuration of the VERSANT HCV genotype assay (Fig. 2A), which is based on the 5′UTR sequences. Another four lines specific for the core region are used for control core amplification and genotypes 6c to 6l, 1a, and 1b, respectively. These core specific lines are only to be considered when 5′UTR line patterns refer to genotype 1.

Upon analysis of the same sample set as described above, the new VERSANT HCV genotype 2.0 assay correctly classified the HCV genotype in 96% (70 of 73) of the samples. All genotype 6 samples could be distinguished from genotype 1 viruses. This result is in agreement with sequence comparisons of HCV core region obtained from these samples, showing differences between genotype 1 and 6 viruses (Fig. 3). Incorporation of core probes in the assay therefore explains the more accurate HCV genotype 6 classification results with the new VERSANT HCV genotype 2.0 assay (Table 1). Only three samples could not be subtyped; the subtype 1a samples Th41 and D89 showed strong binding of core specific line 25 (subtype 1a) but also a faint band at the position of line 26 (subtype 1b), whereas the subtype 3b sample Th48 showed only the UTR-specific line 13 (Fig. 2C).

Three samples (4%) could not be genotyped; sample Th52 (genotype 6 by sequence analysis) showed binding to line 24, indicating a subtype 6 virus, whereas there was no binding to the 5′UTR lines 3 and 4 (Fig. 2C). As shown in Fig. 3, this result can be explained by a single nucleotide substitution in the Th52 sequence at a position where probes 3 and 4 bind (nucleotide 183 [C→T] and nucleotide 215 [A→G], respectively). On the other hand, samples Th6 and Th44 (subtype 1b by sequence analysis) bound to core lines 24 and 26 (genotypes 6 and 1b, Fig. 2C). To test the possibility of double infection in these samples, RT-PCR products of UTR-core regions of Th6 and Th44 samples were cloned, and at least 25 clones per sample were sequenced. No evidence of a mixed genotype infection could be obtained in the samples tested, although the presence of a coinfecting strain as a minor population cannot entirely be ruled out.

DISCUSSION

Clinical studies have shown that the HCV genotype is an important predictor for efficiency of antiviral treatment. Therefore, many efforts have been made to develop assays for HCV genotyping, and several commercial tests are available (13). The gold standard for determination of HCV genotype is sequence analysis of phylogenetically informative coding regions of the HCV genome and comparison to consensus sequences of known genotypes. However, sequence analysis of amplicons is laborious and is not accurate in identifying mixed infections. Moreover, since the informative sequences on the genome are also regions with a relatively high heterogeneity, primers may fail to anneal, resulting in assay failures.

Hybridization of HCV 5′UTR sequences to genotype-specific probes with the VERSANT HCV genotype assay has thus far been the keystone for HCV genotyping because the high degree of sequence conservation makes this region ideal for RT-PCR amplification. However, this region contains lower phylogenetic information than other genomic sequences such as core and NS5b (15). Not surprisingly, this assay's ability to discriminate between HCV genotypes was challenged by the high diversity of HCV sequences, especially by genotypes 6c to 6l from SEA (19, 21, 24; the present study). Because of the high homology of the 5′UTR between genotype 1 and genotypes 6c to 6l, the latter are claimed to be genotype 1 by the assay (24) (Fig. 2 and Table 1). Moreover, it has been reported that the VERSANT HCV genotype assay could not accurately distinguish between genotypes 1a and 1b in 5 to 10% of cases because specific probes for both subtypes are lacking (2). Mistyping of genotype 6 as genotype 1 may influence the clinical management of patients, since genotype 6 viruses show a higher response to therapy than genotype 1 (4).

The new VERSANT HCV genotype 2.0 assay is designed to increase the accuracy of HCV genotype/subtype identification by including core-specific probes that discriminate between genotypes 1a, 1b, and 6c to 6l. Because of the retained phylogenetic information in this region, the core gene is one of the recommended regions for HCV genotyping (18). Although attempts have been made previously to design specific 5′UTR-core probes for genotype identification, these were not tested for genotype 6 viruses (25). In the present study, we tested the new VERSANT HCV genotype 2.0 line probe assay with 73 samples obtained from SEA, of which 45% (33 of 73) were of genotype 6. A marked improvement in accurate genotyping is seen with the new assay compared to the old assay that only targeted the 5′UTR (~96% versus 71%). As expected, the increase in precision is largely due to correct genotyping and subtyping of 6c to 6l and 1a/b viruses, respectively. Our sample set not only contains genotype 6a but also the new variants of genotypes 6d, 6e, 6f, 6i, 6l, and 6n (Fig. 1). All of these were correctly identified by the VERSANT HCV genotype 2.0 assay. With regard to the heterogeneity of genotype 6 sequences, 5′UTR/core line probe assay proved its ability to recognize a broad variety of genotype 6 viruses.

Only 4% (3 of 73) of the samples could not be genotyped. One of these showed a new pattern for genotypes 6c to 6l (Th52). Two other samples (Th6 and Th44) showed specific hybridization with probes of genotypes 1b and 6c to 6l. Although sequence variation between HCV strains offers an explanation since only few nucleotide changes could cause cross-reactivity with probes thought to be subtype specific, this seems not very likely. Alternatively, these results could indicate the presence of genotypically different viruses in the samples. Attempts to confirm this by sequence analysis were not successful, possibly because the detection of mixed infections by sequence analysis of amplified fragments is not sensitive enough (6, 14). The hybridization patterns, indicating a possible mixed infection of genotype 1 and 6 viruses, are not evident from the line patterns observed with the old version of the LiPA test.

One of the other commercial assays that is frequently used for genotyping HCV is the Trugene HCV 5′NC genotyping kit. In comparative studies between this assay and the previous VERSANT HCV genotype test, the accuracy of genotyping was similar, although Trugene showed a slightly higher percentage of correct subtype identifications (5). The efficiency of Trugene in discriminating genotype 6 subtypes is currently not known. However, its exclusive use of the 5′UTR region for genotype identification points toward a reduced efficiency in the correct assignment of genotype 6c to 6l viruses. Therefore, the new VERSANT HCV genotype 2.0 assay, which also takes into account HCV core sequences, may actually be the most suitable current tool for routine HCV genotyping. This assay may not only be crucial for clinical evaluation of patients but also for future epidemiological, evolutionary, and pathogenesis studies.

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